Induction of Apoptosis in Cisplatin-sensitive and -resistant Human Ovarian Cancer Cell Lines¹

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Abstract

In this study, we have assessed the mechanism of cytotoxicity in a series of cisplatin-sensitive and -resistant ovarian carcinoma cells following treatment with equitoxic concentrations of cisplatin. The specific proteolytic degradation and the enzymatic activities of the DNA-dependent protein kinase (DNA-PK) were assessed in the cisplatin-sensitive A2780 cell line and two resistant derivative cell lines, CP70 and C30. Forty-eight h following cisplatin treatment, unattached, apoptotic A2780 cells demonstrated a 20–30% decrease in DNA-PK phosphorylation activity. The resistant CP70 and C30 cell lines showed greater decreases in activity approaching 80 and 90%, respectively. The decreases in kinase activity were attributed to proteolytic degradation of the catalytic subunit of DNA-PK (DNA-PKcs). The extent of degradation mimicked the loss of DNA-PK activity, with the resistant cell lines showing the greatest portion of degraded DNA-PKcs. At the same time point, the ability of the DNA-PK Ku subunits to bind DNA was decreased in apoptotic, unattached cells compared to untreated controls, with the decrease in binding activity being attributed to decreased expression of the Ku subunits. In addition to DNA-PKcs cleavage, specific proteolytic cleavage of the poly(ADP-ribose)-polymerase and generation of nucleosome-length DNA ladders was observed in all cell lines following cisplatin treatment. These data suggest that cell death via the accumulation of cisplatin-damaged DNA occurs via apoptosis in both the cisplatin-resistant and -sensitive ovarian cancer cells.

Introduction

Chemotherapeutic treatment of ovarian cancer often includes cisplatin as part of a combined-drug protocol. Tumor recurrence and broad cross-resistance to unrelated natural products and synthetic chemotherapeutic drugs often occurs (1). Resistance mechanisms include a decrease in cisplatin accumulation within the cell, an enhanced DNA repair mechanism, and an increase in cellular glutathione (2). Cross-resistance to a variety of chemotherapeutic agents with different intercellular targets has led to the hypothesis that a defective apoptotic pathway may be responsible for the resistant phenotype. Support for this hypothesis was obtained in the L1210 murine leukemic cell line that undergoes apoptosis in response to cisplatin treatment, whereas a cisplatin-resistant derivative, L1210/DDP, had a defective apoptotic response to cisplatin treatment (3). Cisplatin-resistant cell lines derived from the human ovarian cancer, A2780, show a 2–3-fold increased removal/repair of cisplatin-DNA adducts, and resistance was correlated to cellular tolerance to cisplatin (4). Interestingly, a recent study comparing unrelated human ovarian cancer cell lines also correlated tolerance to cisplatin resistance (5). It was hypothesized that increased tolerance may be the result of a defective apoptotic process such that increased levels of DNA damage are required to induce the signal initiating apoptosis.

Materials and Methods

Materials. Cisplatin was from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were from Life Technologies, Inc. (Grand Island, NY). Antibodies to the DNA-PKcs (rabbit polyclonal DP1K IgG) were a gift from Dr. S. P. Lees-Miller (University of Calgary, Calgary, Alberta, Canada). Antibodies to the p70 and p80 subunits of Ku were kindly supplied by Dr. W. Reeves (University of North Carolina, Chapel Hill, NC). Anti-PARP, C-terminal rabbit polyclonal antibody was purchased from BioRad Research Laboratories, Inc. (Plymouth Meeting, PA). Goat anti-rabbit IgG HRP was purchased from Bio-Rad Laboratories (Hercules, CA). Goat anti-mouse IgG HRP was purchased from Life Technologies, Inc. Renaissance Western blot chemiluminescent reagents were purchased from NEN Life Science Products (Boston, MA). The ovarian cancer cell lines, A2780, CP70, and C30 were a gift from Dr. T. C. Hamilton (Fox Chase Cancer, Philadelphia, PA). All other chemicals were obtained through standard suppliers.

Cell Culture. The human ovarian cancer cell lines, HeLa, A2780, CP70, and C30, were maintained in monolayer cultures in DMEM containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate in a 37°C humidified environment with 5% CO₂. For cytotoxicity data, clonogenic analysis was used. Five hundred cells from each cell line were plated into 60-mm tissue culture dishes 24 h prior to drug treatment. Cells were treated in triplicate for 4 h, after which cisplatin-containing medium was removed, and fresh medium was added to the cells. Incubation was continued until colonies of approximately 50 cells were observed (7–14 days). Viability was determined by staining with 1% methylene blue in 70% ethanol, followed by three washes in deionized water. Data obtained were used to calculate cisplatin IC₅₀ and IC₉₀ values for each cell line. For all experiments, 3 X 10⁴ cells from each cell line were plated into 150-mm tissue culture dishes 24 h prior to cisplatin treatment. Cells were either untreated or treated with cisplatin at the corresponding IC₅₀ and IC₉₀ level for 4 h at 37°C. Plates were washed three times with PBS, and 10 ml of fresh medium was added. Cells were incubated for an additional 48 h, at which time cells were collected.

A conserved feature of the apoptotic process is the specific degradation of a series of proteins by the aspartate-specific cysteine proteinases or caspasases (6, 7). One target for apoptotic proteolytic degradation is DNA-PKcs (8, 9). Ku, the DNA binding subunits of DNA-PK, remains stable and experiences no cleavage of either subunits during apoptosis (8, 10). Our laboratory has been interested in proteins that bind to damaged DNA and how they can alter the repair pathways. We identified DNA-PK as being capable of binding cisplatin-damaged DNA, but once bound, kinase activity is abrogated (11, 12). Binding of Ku to double-strand DNA ends, nicks, or larger gaps (13, 14) is essential in vitro to activate the kinase activity of DNA-PK to phosphorylate itself or other transcription factors. In this report, we assessed the induction of apoptosis in A2780, CP70, and C30 cell lines in response to cisplatin treatment. By assessing the cleavage of DNA-PKcs and measurement of DNA-PK and Ku activities and DNA fragmentation, we demonstrate that cisplatin resistance in CP70 and C30 ovarian cell lines is not the result of an inability to activate the apoptotic pathway.

¹ The abbreviations used are: DNA-PKcs, catalytic subunit of the DNA-PK; DNA-PK, DNA-dependent protein kinase; PARP, poly(ADP-ribose)polymerase; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase.
Nonadherent cells present in the medium were removed from the monolayer or adherent, live cells were collected separately via scraping. All cells were collected by sedimenting at 2000 x g for 5 min.

**Preparation of Cell-free Extracts.** Protein was extracted from cells based on a modification of the procedure of Wood et al. (15). Cell pellets were washed twice with PBS and resuspended in wash buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM MgCl₂, and 1 mM β-mercaptoethanol]. Cells were collected by sedimentation, quick frozen in liquid nitrogen, and slow thawed on ice. Cell pellets were resuspended in lysis buffer [10 mM Tris-Cl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol, and 0.5% CHAPSO] and were incubated on ice for 30 min. An equal volume of high salt buffer [50 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 2 mM DTT, 25% sucrose, and 50% glycerol] was added, and the lysate was vortexed briefly. Saturated ammonium sulfate was added to 15%, and incubation continued on ice for an additional 30 min. Insoluble material was removed by sedimentation at 14,000 x g for 30 min at 4°C. Supernatants were dialyzed against 500 ml of dialysis buffer [25 mM HEPES (pH 7.8), 1 mM EGTA, 0.01% NP40, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM M KCl] supplemented with 1 μg/ml of each leupeptin and pepstatin for 2 h at 4°C. Protein concentrations of extracts were obtained using the microassay protocol of the Bio-Rad protein assay reagent with BSA as standards. Cell-free extracts were frozen at -70°C until needed.

**Extraction of DNA for the Determination of DNA Fragmentation.** Cells (3 x 10⁶) from each cell line were either untreated or treated with cisplatin at their respective 4-h cisplatin IC₅₀ and IC₉₀ concentrations. Drug was removed from cells and replaced with fresh media, and cells were allowed to continue in culture for 48 h, at which time only adherent, untreated or nonadherent, treated cells were sedimented at 2500 rpm. DNA was extracted and analyzed for fragmentation according to Wolfe et al. (16).

**SDS-PAGE/Western Blot Analysis.** SDS-PAGE was performed essentially according to Lammeli (17). Twenty μg of protein per lane were separated on 8 x 10-cm mini-SDS gels comprised of an 8% separation and a 4% stacker gel. Electrophoresis was carried out at 125 V through the stacker gel and then 250 V for 1.5 h. Electrophoretic transfer to Immobilon P membranes (Millipore, Bedford, MA) was performed at 350 mA for 3 h at 4°C in 10 mM 3-(cyclohexylamino) propane sulfonic acid in 10% methanol (pH 10.5). Filters were blocked in 2% nonfat dry milk, 0.2% Tween 20 in PBS for 1 h at room temperature. Membranes were then incubated for 1 h at room temperature with the primary antibody dilution noted in the figure legends. Membranes were then washed three times and incubated for 1 h with the peroxidase conjugated secondary antibody dilution noted in the figure legends. Membranes were washed again, and peroxidase was visualized using the Renaissance chemiluminescent reagent kit, per the instructions of NEN Life Science Products.

**Enzyme Assays.** DNA-PK assays were performed according to our procedure published previously (12). Briefly, assays measured the transfer of [γ-³²P]ATP to the DNA-specific synthetic peptide (EPPLSQAEAFDLWKK) dependent on DNA. EMSAs were used to determine Ku binding activity in the cell-free extracts similar to our procedure published previously (18). Briefly, mobility shift assays measured binding of the Ku subunits to 50 fmol of ³²P-labeled 75-mer DNA substrate. Reaction products were separated by 8% native PAGE at 150 V for 2 h. Gels were dried, and products were visualized by autoradiography and quantified by a Molecular Dynamics PhosphorImager using ImageQuant software in the volume integration mode.

**Results and Discussion**

**Cisplatin Toxicity.** The well-characterized ovarian carcinoma cell line A2780 and its resistant derivatives CP70 and C30 were chosen to assess the induction of apoptosis in response to cisplatin treatment. A variety of cytotoxicity data have been reported for these cell lines (4, 5), and considering the variation in the published results, we assessed the response of each cell line to cisplatin and calculated IC₅₀ and IC₉₀ values. These data are presented in Table 1 and reveal a 9-10- and 25-30-fold resistance for the CP70 and C30 cell lines, respectively. These values are similar to those reported previously for these cell lines, with each cell line displaying slightly higher levels of resistance to cisplatin. We also assessed the relative toxicity to camptothecin, and a similar cross-resistance was observed for the CP70 and C30 cells lines (data not shown).

**Effect of Equitoxic Cisplatin Treatment on DNA-PK and DNA Binding Activity.** We have assessed the expression and activities of DNA-PK in response to equitoxic treatment of cells with cisplatin. DNA-PK is a target for caspase catalyzed degradation in the apoptotic pathway (8, 9). The kinase activity of DNA-PK was assessed in vitro using cell-free extracts prepared from untreated control cells and cells collected from culture supernatants 48 h following cisplatin treatment. The results presented in Fig. 1A demonstrate that all three cell lines exhibit a decrease in DNA-PK phosphorylation activity in response to cisplatin. Interestingly, both the CP70 and C30 cell lines had less DNA-PK activity in untreated controls compared to the A2780 cell line. Compared to each untreated control (open bars), the decreases seen in cells treated at the IC₅₀ levels (closed bars) reach approximately 16, 53, and 82% for the A2780, CP70, and C30 cell lines, respectively. Treatment at the IC₉₀ levels (closed bars) resulted in 24, 71, and 91% inhibition for A2780, CP70, and C30 cell lines, respectively. These results demonstrate that cisplatin treatment results in a decrease in DNA-PK activity independent of whether cells are sensitive or resistant to cisplatin.

### Table 1 Sensitivity of A2780 and its resistant mutants to cisplatin at 4-h exposure times

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin IC₅₀ (μM)</th>
<th>Relative resistance</th>
<th>Cisplatin IC₉₀ (μM)</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>1.92</td>
<td>1.0</td>
<td>7.85</td>
<td>1.0</td>
</tr>
<tr>
<td>CP70</td>
<td>18.00</td>
<td>9.4</td>
<td>80.08</td>
<td>10.1</td>
</tr>
<tr>
<td>C30</td>
<td>56.77</td>
<td>29.6</td>
<td>210.76</td>
<td>26.8</td>
</tr>
</tbody>
</table>

*The IC₅₀ and IC₉₀ cisplatin concentrations, as determined, are the levels of drug that inhibited cell growth by 50 and 90%, respectively. These values are the result of triplicate determinations.
The DNA-binding subunits of DNA-PK, Ku, were assessed for the ability to bind duplex DNA in the same cell extracts. Quantification of the EMSA analyses are presented in Fig. 1B and demonstrate that, similar to the kinase activity of DNA-PK, a lower level of Ku binding activity was observed in untreated CP70 and C30 cell lines compared to the A2780 cells. Similarly, in response to cisplatin treatment at the IC_{50} and IC_{90} levels for each cell line, a decrease in Ku DNA binding activity was observed. The results presented reveal that the A2780 cells retained the most Ku DNA binding activity following cisplatin treatment, and C30 retained the least.

**Effect of Equitoxic Cisplatin Treatment on DNA-PK Subunit Expression.** Considering the results demonstrating a decrease in Ku DNA binding activity, a possible explanation for these data is the decrease in DNA-PK kinase activity could be the result of a decrease in Ku subunit expression or apoptosis-induced degradation of the DNA-PKcs subunit. To determine how the decreases in both the kinase catalytic activity and the DNA binding activity are manifested, we assessed protein expression by Western blot analysis. The catalytic subunit of DNA-PK is a substrate for caspase cleavage generating a Mr 240,000 inactive proteolytic product from the Mr 460,000 full-length protein (8, 9). The Western blot results detecting DNA-PKcs in untreated control cells and treated unadherent cells is presented in Fig. 2A. Lane 1 is a positive control lane containing fully active DNA-PK purified from HeLa cells, which migrates at Mr 460,000 (11). HeLa cells were also used as a control for the protein extraction procedure and reveal full-length DNA-PKcs in the absence of treatment (Fig. 2A, Lane 2). Similar results were observed in the A2780, CP70, and C30 cells lines (Fig. 2A, Lanes 3–5). When cells were treated with cisplatin at their respective IC_{50} concentrations, a decrease in the amount of full-length DNA-PKcs is apparent, and the appearance of the Mr 240,000 fragment is evident in extracts derived from nonadherent, apoptotic cells (Fig. 2A, Lanes 6–8). The specific cleavage is observed in all cells, regardless of cellular cisplatin sensitivity or resistance. The degree of proteolytic cleavage correlated with the decrease in DNA-PK activity. A2780 cells retained the most activity following treatment, as well as the most full-length DNA-PKcs. Approximately 50% of the DNA-PKcs in the A2780 cells was retained as the full-length unproteolyzed enzyme. Similar results were obtained when cells were treated at their IC_{90} cisplatin concentrations (data not shown). An additional antibody-reactive protein is present in extracts from the C30 cell line at Mr 150,000, regardless of cisplatin exposure (Fig. 2A, compare Lanes 5 and 8). A product of this size was shown to be an additional DNA-PKcs cleavage fragment in Burkitt’s lymphoma cells treated with etoposide (8, 10). The appearance of this fragment in untreated C30 cell extracts, although not in A2780 or CP70 extracts, is currently under investigation.

The effect of cisplatin-induced apoptosis on the Ku autoantigen from these same cell-free extracts was analyzed using mouse monoclonal antibodies directed against the p80 and p70 Ku subunits. As shown in Fig. 2B, when compared directly to the Ku detected in untreated, adherent cells (Lanes 3–5), the p70 and p80 subunits of Ku remain fully intact in all cell lines at cisplatin concentrations yielding 50% (Lanes 6–8) and 90% cell death (data not shown). The Western blot evidence demonstrating no specific proteolytic cleavage of Ku in these cell-free extracts is consistent with previous reports (8, 9). A small decrease in expression levels is apparent in the cisplatin-treated A2780 cell line compared to untreated controls. Interestingly, the amount of p70 and p80 reactive protein is decreased in extracts from the treated CP70 and C30 cell lines. These data are contrary to previous results and may be the result of the time frame of the different experiments. Our protocol measured protein expression at 48 h after treatment, whereas the earlier report assessed Ku at 1–5 h following treatment (8).

**Apoptotic Cleavage of PARP.** The DNA-PKcs cleavage suggests that in fact apoptosis is being induced in each cell line. These results demonstrate that resistant CP70 and C30 cells have an intact, functional apoptotic pathway inducible by cisplatin. Additional evidence to support the occurrence of apoptosis in these human ovarian tumor cells is provided by the proteolytic cleavage of PARP. PARP is a Mr 116,000 enzyme responsible for the poly(ADP)-ribosylation of itself or other proteins after exposure to single- or double-stranded nicked DNA (19). PARP is cleaved at a specific aspartic acid residue by the CCP32 protease (20), similar to DNA-PKcs. Cleavage results in the full-length Mr 116,000 enzyme being converted to a Mr 85,000 form (21), which retains the C-terminal catalytic and automodification activities, albeit to a lesser degree than that of the fully intact PARP molecule (22).

The results of Western blot analysis from A2780, CP70, and C30 cell lines is presented in Fig. 3A. Extracts from untreated, adherent cells containing only the full-length Mr 116,000 PARP are shown in Lanes 1–3. Specific cleavage to the Mr 85,000 form is apparent...
following cisplatin treatment (Lanes 4–6). Similar results were achieved when cells were treated at their respective cisplatin IC50 concentrations (data not shown). In both of the cisplatin-resistant cell lines, PARP is completely degraded upon exposure to equitoxic concentrations of cisplatin, similar to the degree of DNA-PKcs degraded in these same cells, as evidenced in Fig. 2A.

DNA Fragmentation in Response to Cisplatin. The data reported thus far rely on caspase degradation of specific proteins to demonstrate a functioning apoptotic pathway in the human ovarian cancer cell lines. A second well-conserved feature of apoptosis is nucleosome DNA fragmentation. Therefore, DNA was isolated from adherent untreated control cells (Fig. 3B, Lanes 1–3) and cells treated at the IC50 (Fig. 3C, Lanes 1–3) and IC100 (Fig. 3C, Lanes 4–6) levels and then separated by agarose gel electrophoresis. The result demonstrates that DNA from untreated cells remains in a high molecular weight form (3B, Lanes 1–3). However, following cisplatin treatment, DNA degradation is observed in each cell line (Fig. 3C). Treatment at the IC50 level resulted in a ladder of DNA products in both the control CP70 and C30 cell lines (Lanes 2 and 3). Cisplatin treatment at the IC100 value resulted in a greater degree of DNA degradation in each of the cell lines (Lanes 4–6). Treatment of A2780 cells at the highest cisplatin concentration revealed DNA nucleosome length DNA degradation (Lane 4). Overall, the degree of DNA degradation was similar to the results observed measuring specific protein degradation. A2780 cells exhibited the least degree of degradation and retained a greater portion of high molecular weight DNA. DNA degradation in both the control CP70 and C30 cell lines was considerably more apparent, generating DNA fragments at 200-bp intervals.

The results presented in this study demonstrate, via DNA degradation and caspase cleavage of specific target proteins, that resistant subclones from an ovarian carcinoma cell line have a functional apoptotic pathway in response to cisplatin. These results argue against the resistance mechanism in CP70 and C30 cells being caused by a defective apoptotic process, as has been shown in other cisplatin-resistant cell lines (3). Interestingly, the apparent degree of apoptosis induction in response to equitoxic doses of cisplatin does vary between the sensitive parental cells and resistant subclones, with the sensitive parental cell line inducing apoptosis to a lesser extent than either of the two resistant subclones. These data support the hypothesis that the mechanism of cisplatin resistance in CP70 and C30 cells is an upstream event that signals the initiation of apoptosis but not in the apoptotic process itself.

References
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